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APPLICATION NUMBER: *60/510,057*
FILING DATE: *October 09, 2003*
RELATED PCT APPLICATION NUMBER: *PCT/US04/33178*

Certified by

Jon W. Dudas

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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

Express Mail Label No. EL 934999135 US

INVENTOR(S)

Given Name (first and middle [if any])	Family Name or Surname	Residence (City and either State or Foreign Country)
Kenneth	Walsh	Boston, MA
Noriyuki	Ouchi	Boston, MA

Additional inventors are being named on the _____ *separately numbered sheets attached hereto*

TITLE OF THE INVENTION (500 characters max)

Method and Composition for Stimulation of Angiogenesis

Direct all correspondence to: **CORRESPONDENCE ADDRESS**

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OR

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ENCLOSED APPLICATION PARTS *(check all that apply)*

[X] Specification *Number of Pages* 31

[X] Drawing(s) *Number of Sheets* 7

[X] Application Data Sheet. See 37 CFR 1.76

[ ] CD(s), Number _____

[X] Other (specify) Express Mail Cert.; Fee Transmittal; Check - $80.00; Return Postcard

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[X] Applicant claims small entity status. See 37 CFR 1.27.

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[ ] No.

[X] Yes, the name of the U.S. Government agency and the Government contract number are: AR40197, HD23681, AG17241 and AG15052

Respectfully submitted,

SIGNATURE _____

Date 10/9/03

REGISTRATION NO. 34,235 *(if appropriate)*

Docket Number: 701586-054550-P

TYPED or PRINTED NAME David S. Resnick

TELEPHONE 617-345-6057

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Practitioner's Docket No. 701586-054550-P *PATENT*

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: Kenneth Walsh and Noriyuki Ouchi

Application No.: Not assigned Group No.:
Filed: Herewith Examiner:
For: METHOD AND COMPOSITION FOR STIMULATION OF ANGIOGENESIS

Mail Stop Provisional Patent Application
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1. Provisional Application for Cover Sheet (1pg.);
2. Provisional Patent Application (38 pp.): Specification (29 pp.); Claims (1 pg.); Abstract (1 pg.); and Drawings (7 pp.);
3. Fee Transmittal for FY 2003 (1 pg.);
4. Check in the amount of $80.00;
5. Application Data Sheet (2 pp.);
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☒ Applicant claims small entity status. See 37 CFR 1.27

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Complete if Known	
Application Number	To be assigned
Filing Date	To be assigned
First Named Inventor	Kenneth Walsh and Noriyuki Ouchi
Examiner Name	To be assigned
Art Unit	To be assigned
Attorney Docket No.	701586-054550-P

METHOD OF PAYMENT *(check all that apply)*

☒ Check ☐ Credit card ☐ Money Order ☐ Other ☐ None

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FEE CALCULATION

1. BASIC FILING FEE

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description	Fee Paid
1001	750	2001	375	Utility filing fee	
1002	330	2002	165	Design filing fee	
1003	520	2003	260	Plant filing fee	
1004	750	2004	375	Reissue filing fee	
1005	160	2005	80	Provisional filing fee	$80.00

SUBTOTAL (1) ($) 80.00

2. EXTRA CLAIM FEES FOR UTILITY AND REISSUE

		Extra Claims		Fee from below		Fee Paid
Total Claims		-20** =		X	=	
Independent Claims		- 3** =		X	=	
Multiple Dependent					=	

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description
1202	18	2202	9	Claims in excess of 20
1201	84	2201	42	Independent claims in excess of 3
1203	280	2203	140	Multiple dependent claim, if not paid
1204	84	2204	42	** Reissue independent claims over original patent
1205	18	2205	9	** Reissue claims in excess of 20 and over original patent

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FEE CALCULATION (continued)

3. ADDITIONAL FEES

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1051	130	2051	65	Surcharge - late filing fee or oath	
1052	50	2052	25	Surcharge - late provisional filing fee or cover sheet	
1053	130	1053	130	Non-English specification	
1812	2,520	1812	2,520	For filing a request for *ex parte* reexamination	
1804	920*	1804	920*	Requesting publication of SIR prior to Examiner action	
1805	1,840*	1805	1,840*	Requesting publication of SIR after Examiner action	
1251	110	2251	55	Extension for reply within first month	
1252	410	2252	205	Extension for reply within second month	
1253	930	2253	465	Extension for reply within third month	
1254	1,450	2254	725	Extension for reply within fourth month	
1255	1,970	2255	985	Extension for reply within fifth month	
1401	320	2401	160	Notice of Appeal	
1402	320	2402	160	Filing brief in support of an appeal	
1403	280	2403	140	Request for oral hearing	
1451	1,510	1451	1,510	Petition to institute a public use proceeding	
1452	110	2452	55	Petition to revive - unavoidable	
1453	1,300	2453	650	Petition to revive - unintentional	
1501	1,300	2501	650	Utility issue fee (or reissue)	
1502	470	2502	235	Design issue fee	
1503	630	2503	315	Plant issue fee	
1460	130	1460	130	Petitions to the Commissioner	
1807	50	1807	50	Processing fee under 37 CFR 1.17(q)	
1806	180	1806	180	Submission of Information Disclosure Stmt	
8021	40	8021	40	Recording each patent assignment per property (times number of properties)	
1809	750	2809	375	Filing a submission after final rejection (37 CFR 1.129(a))	
1810	750	2810	375	For each additional invention to be examined (37 CFR 1.129(b))	
1801	750	2801	375	Request for Continued Examination (RCE)	
1802	900	1802	900	Request for expedited examination of a design application	

Other fee (specify) _____

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SUBTOTAL (3) ($) 80.00

SUBMITTED BY

(Complete *(if applicable)*)

Name (Print/Type)	David S. Resnick	Registration No. (Attorney/Agent)	34,235	Telephone	617-345-6057
Signature				Date	10/9/03

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Docket No.: 701586-054550
Express Mail No. EL 934999135 US

METHOD AND COMPOSITION FOR STIMULATION OF ANGIOGENESIS

[0001] This invention was made in part with U.S. Government support under Contact Numbers AR40197, HD23681, AG17241, and AG15052 awarded by the National Institutes of Health. The U.S. Government has certain rights in this application.

FIELD OF THE INVENTION

[0002] The present invention provides for novel pharmaceutical compositions, and methods of use thereof for treatment of diseases or disorders involving angiogenesis.

BACKGROUND OF THE INVENTION

[0003] Blood vessels are the means by which oxygen and nutrients are supplied to living tissues and waste products are removed from living tissue. Angiogenesis refers to the process by which new blood vessels are formed. (46; reviewed by Folkman, J., 2001, Semin. Oncol. 28 (6): 536-42; Ribatti, D., et al., 2000, Gen. Pharmacol. 35 (5): 227-31). Thus, where appropriate, angiogenesis is a critical biological process. It is essential in reproduction, development and wound repair. However, inappropriate angiogenesis can have severe negative consequences. For example, it is only after many solid tumors are vascularized as a result of angiogenesis that the tumors have a sufficient supply of oxygen and nutrients that permit it to grow rapidly and metastasize. Because maintaining the rate of angiogenesis in its proper equilibrium is so critical to a range of functions, it must be carefully regulated in order to maintain health. The angiogenesis process is believed to begin with the degradation of the basement membrane by proteases secreted from endothelial cells (EC) activated by mitogens such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF). The cells migrate and proliferate, leading to the formation of solid endothelial cell sprouts into

the stromal space, then, vascular loops are formed and capillary tubes develop with formation of tight junctions and deposition of new basement membrane.

[0004] In adults, the proliferation rate of endothelial cells is typically low compared to other cell types in the body. The turnover time of these cells can exceed one thousand days. Physiological exceptions in which angiogenesis results in rapid proliferation typically occurs under tight regulation, such as found in the female reproductive system and during wound healing.

[0005] The rate of angiogenesis involves a change in the local equilibrium between positive and negative regulators of the growth of microvessels. The therapeutic implications of angiogenic growth factors were first described by Folkman and colleagues over two decades ago (47). Abnormal angiogenesis occurs when there are increased or decreased stimuli for angiogenesis resulting in either excessive or insufficient blood vessel growth, respectively. For instance, conditions such as ulcers, strokes, and heart attacks may result from the absence of angiogenesis normally required for natural healing.

[0006] Thus, there are instances where a greater degree of angiogenesis is desirable-- increasing blood circulation, wound healing, and ulcer healing. For example, investigations have established the feasibility of using recombinant angiogenic growth factors, such as fibroblast growth factor (FGF) family (48, 49), endothelial cell growth factor (ECGF) (50), and more recently, vascular endothelial growth factor (VEGF) to expedite and/or augment collateral artery development in animal models of myocardial and hindlimb ischemia (50, 51).

[0007] Although preliminary results with the angiogenic proteins are promising, new angiogenic agents that show improvement in size, ease of production, stability and/or potency would be desirable.

SUMMARY OF THE INVENTION

[0008] We have surprisingly discovered that adiponectin, an adipocyte specific cytokine, regulates angiogenesis. As a result of this discovery, the present invention provides for stimulation of angiogenesis with adiponectin.

[0009] The present invention further provides compositions and methods for stimulating angiogenesis in a tissue associated with a disease condition. A composition

comprising an angiogenesis-stimulating amount of adiponectin protein is administered to tissue to be treated for a disease condition that responds to new blood vessel formation.

[00010] The composition providing the adiponectin protein can contain purified protein, biologically active protein fragments, recombinantly produced adiponectin protein or protein fragments or fusion proteins, or gene/nucleic acid expression vectors for expressing adiponectin protein.

[00011] The present invention can be used alone or in combination with other strategies to stimulate angiogenesis.

[00012] The tissue to be treated can be any tissue in which potentiation of angiogenesis is desirable. For example, adiponectin is useful to treat patients with hypoxic tissues such as those following stroke, myocardial infarction or associated with chronic ulcers, tissues in patients with ischemic limbs in which there is abnormal, i.e., poor circulation, due to diabetic or other conditions. Patients with chronic wounds that do not heal, and therefore could benefit from the increase in vascular cell proliferation and neovascularization, can be treated as well. Potentiation of angiogenesis would also offer therapeutic benefit for ischemic vascular diseases, including coronary artery insufficiency and ischemic cardiomyopathy, peripheral arterial occlusive disease, cerebrovascular disease, ischemic bowel syndromes, impotence, and would healing.

[00013] The adiponectin protein, peptide, and nucleic acid sequence encoding adiponectin protein or peptide may be administered in conjunction with another angiogenesis stimulator.

[00014] The present invention also encompasses a pharmaceutical composition suitable for stimulating angiogenesis in a target mammalian tissue comprising a viral or non-viral gene transfer vector containing a nucleic acid, the nucleic acid having a nucleic acid segment encoding for adiponectin protein or peptide, and a pharmaceutically acceptable carrier.

[00015] Other aspects of the invention are disclosed *infra*.

BRIEF DESCRIPTION OF THE DRAWINGS

[00016] Figure 1 shows that adiponectin promotes endothelial cell migration and differentiation into tube-like structures. Tube formation assays were performed (A and B).

HUVECs were seeded on Matrigel-coated culture dishes in the presence of adiponectin (30 μg/ml), VEGF (20 ng/ml) or BSA (30 μg/ml)(Control). A) Representative cultures are shown. B) Quantitative analysis of tube formation. C) A modified Boyden chamber assay was performed using HUVECs. HUVECs were treated with adiponectin (30 μg/ml), VEGF (20 ng/ml) or BSA (30 μg/ml)(Control). Results are show as the mean ± SE. Results are expressed relative to the values compared to control. *$p<0.01$ vs. control.

[00017] Figure 2 shows adiponectin-stimulated signaling in endothelial cells. A) Time-dependent changes in the phosphorylation of AMPK, Akt, eNOS and ERK following adiponectin treatment (30 μg/ml). B) Role of AMPK in the regulation of adiponectin-induced protein phosphorylation. HUVECs were transduced with an adenoviral vector expressing dominant-negative AMPK tagged with c-Myc (dn-AMPK) or an adenoviral vector expressing GFP (Control) 24 h before serum-starvation. After 16-h serum-starvation, cells were treated with adiponectin (30 μg/ml) for the indicated lengths of time. C) Role of Akt in the regulation of adiponectin-induced protein phosphorylation. HUVECs were transduced with an adenoviral vector expressing dominant-negative Akt (dn-Akt) or an adenoviral vector expressing GFP (Control) 24 h before serum-starvation. After 16-h serum-starvation, cells were treated with adiponectin (30 μg/ml) for the indicated lengths of time. Representative blots are shown.

[00018] Figure 3 shows the contribution of AMPK and Akt to adiponectin-induced angiogenic cellular responses. HUVECs were transduced with an adenoviral vector expressing dn-AMPK (hatch), dn-Akt (open) or GFP (Control, solid) 24 h before the change to low-serum media. After 16-h serum-starvation, in vitro Matrigel (A, B) or modified Boyden chamber assays (C) were performed. Cells were treated with adiponectin (30 μg/ml) or BSA (30 μg/ml)(Vehicle). A) Representative cultures displaying tube formation are shown. B) Quantitative analysis of tube lengths. C) Modified Boyden chamber assay was performed with adiponectin or VEGF as chemoattractant. Results are shown as the mean ± SE. Results are expressed relative to the values compared to control. *$p<0.01$ vs. each control.

[00019] Figure 4 shows that PI3-kinase signaling is involved in adiponectin-induced angiogenic pathway. A) Quantitative analysis of tube formation is shown. HUVECs were treated with adiponectin (30 μg/ml) or BSA (30 μg/ml) in the presence of LY294002 (10 μM) or vehicle at the time seeding. B) A modified Boyden chamber assay was performed using

adiponectin as the chemoattractant. HUVECs were pretreated with LY294002 (10 μM) or vehicle for 1 h and then incubated with adiponectin (30 μg/ml) or BSA (30 μg/ml) for 4 h. C) Effects of LY294002 on adiponectin-stimulated protein phosphorylation. Representative blots are shown. HUVECs were pretreated with LY294002 (10 μM) or vehicle for 1 h and then incubated with adiponectin (30 μg/ml) or BSA (30 μg/ml) for the indicated lengths of time. Results are presented as the mean ± SE. For A and B, results are expressed relative to the values compared to control. *, $p<0.01$.

[00020] Figure 5 shows that adiponectin promotes angiogenesis in vivo. An in vivo Matrigel plug assay was performed to evaluate the effect of adiponectin on angiogenesis (A and B). Matrigel plugs containing adiponectin (100 μg/ml, n=3) or PBS (Control, n=3) were injected subcutaneously into mice. A) Plugs were stained with the endothelial cell marker CD31. Bar: 100 μm. B) The frequency of CD31-positive cells in five low power fields was determined for each Matrigel plug. Data were presented as fold increase of CD31-positive cells relative to the control. Rabbit cornea assay was performed (C and D). Pellets containing adiponectin (1 μg and 10 μg, n=8), VEGF (100 ng, n=8) or PBS (Control, n=8) were implanted in the cornea. C) Photographs of rabbit eyes are shown (Control, adiponectin 10 μg, VEGF 100 ng). D) An angiogenic score was calculated (vessel density x distance from limbus). Results are shown as the mean ± SE. *$P<0.01$ vs. control.

[00021] Figure 6 shows a proposed scheme for adiponectin-stimulated signaling in endothelial cells. Adiponectin activates AMPK which, in turn, promotes Akt activation, eNOS phosphorylation and angiogenesis. PI3-kinase is essential for adiponectin-mediated activation of Akt. Both AMPK and Akt can directly phosphorylate eNOS. However, inhibition of Akt or PI3-kinase was found to suppress adiponectin-stimulated eNOS phosphorylation without interfering with AMPK activation. Therefore, the data are most consistent with an AMPK-PI3-kinase-Akt-eNOS signaling axis.

DETAILED DESCRIPTION OF THE INVENTION

[00022] The present inventors have discovered that adiponectin promotes angiogenesis through activation of AMPK- and phosphatidylinositol-3-kinase (PI3-kinase)-AKT-dependent pathways in endothelial cells.

[00023] This discovery is important because of the role that angiogenesis plays in a variety of disease processes. In addition, injured tissue requires angiogenesis for tissue growth and it is desirable to potentiate or promote angiogenesis and to promote tissue healing and growth. For example, adiponectin can be used to treat patients with ischemic limbs in which there is abnormal, i.e. poor circulation as a result of diabetes or other conditions. Also contemplated are patients with chronic wounds which do not heal and therefore could benefit from the increase in vascular cell proliferation and neovascularization.

[00024] Adiponectin protein useful in the present invention can be produced in any of a variety of methods including isolation from natural sources including tissue, production by recombinant DNA expression and purification, and the like. Adiponectin protein can also be provided "in situ" by introduction of a gene therapy system to the tissue of interest which then expresses the protein in the tissue.

[00025] A gene encoding adiponectin protein can be prepared by a variety of methods known in the art. For example, the gene can readily be cloned using cDNA cloning methods from any tissue expressing the protein. The accession number for the human adiponectin gene transcript is NM_004797 and the rat accession number is NM_144744. Protein accession numbers are NP_004788 and NP_653345 for human and rat respectively. See also, US 5,869,330; US20020132773; US200230147855 and US200230176328.

[00026] The nucleotide sequences of particular use in the present invention, which, encode for adiponectin protein, include various DNA segments, recombinant DNA (rDNA) molecules and vectors constructed for expression of adiponectin protein. DNA molecules (segments) of this invention therefore can comprise sequences which encode whole structural genes, fragments of structural genes, and transcription units.

[00027] A preferred DNA segment is a nucleotide sequence which encodes adiponectin protein as defined herein, or biologically active fragment thereof. By biologically active, it is meant that the expressed protein will have at least some of the biological activity of the intact protein found in a cell.

[00028] A preferred DNA segment codes for an amino acid residue sequence substantially the same as, and preferably consisting essentially of, an amino acid residue sequence or portions thereof corresponding to adiponectin protein described herein.

[00029] A nucleic acid is any polynucleotide or nucleic acid fragment, whether it be a polyribonucleotide of polydeoxyribonucleotide, i.e., RNA or DNA, or analogs thereof.

[00030] DNA segments are produced by a number of means including chemical synthesis methods and recombinant approaches, preferably by cloning or by polymerase chain reaction (PCR).

[00031] The adiponectin gene of this invention can be cloned from a suitable source of genomic DNA or messenger RNA (mRNA) by a variety of biochemical methods. Cloning these genes can be conducted according to the general methods known in the art. Sources of nucleic acids for cloning an adiponectin gene suitable for use in the methods of this invention can include genomic DNA or messenger RNA (mRNA) in the form of a cDNA library, from a tissue believed to express these proteins.

[00032] A preferred cloning method involves the preparation of a cDNA library using standard methods, and isolating the adiponectin-encoding or nucleotide sequence by PCR amplification using paired oligonucleotide primers based on nucleotide sequences described herein. Alternatively, the desired cDNA clones can be identified and isolated from a cDNA or genomic library by conventional nucleic acid hybridization methods using a hybridization probe based on the nucleic acid sequences described herein. Other methods of isolating and cloning suitable adiponectin-encoding nucleic acids are readily apparent to one skilled in the art.

[00033] The invention also includes a recombinant DNA molecule (rDNA)containing a DNA segment encoding adiponectin as described herein. An expressible rDNA can be produced by operatively (in frame, expressibly) linking a vector to an adiponectin encoding DNA segment of the present invention.

[00034] The choice of vector to which a DNA segment of the present invention is operatively linked depends directly, as is well known in the art, on the functional properties desired, e.g., protein expression, and the host cell to be transformed. Both prokaryotic and eukaryotic expression vectors are familiar to one of ordinary skill in the art of vector construction, and are described by Sambrook et al., Molecular Cloning: A Laboratory Manual Cold Spring Harbor Laboratory (2001).

[00035] Expression vectors compatible with eukaryotic cells, preferably those compatible with vertebrate cells, can be used to form the recombinant DNA molecules of the

present invention. Eukaryotic cell expression vectors are well known in the art and are available from several commercial sources. Typically, such vectors are provided containing convenient restriction sites for insertion of the desired DNA segment.

[00036] Additionally, the angiogenesis modulator can also be delivered using gene therapy. The gene transfer methods for gene therapy fall into three broad categories: (1) physical (e.g., electroporation, direct gene transfer and particle bombardment), (2) chemical (e.g. lipid-based carriers and other non-viral vectors) and (3) biological (e.g. virus derived vectors). For example, non-viral vectors such as liposomes coated with DNA may be directly injected intravenously into the patient. It is believed that the liposome/DNA complexes are concentrated in the liver where they deliver the DNA to macrophages and Kupffer cells.

[00037] Gene therapy methodologies can also be described by delivery site. Fundamental ways to deliver genes include ex vivo gene transfer, in vivo gene transfer, and in vitro gene transfer. In ex vivo gene transfer, cells are taken from the patient and grown in cell culture. The DNA is transfected into the cells, the transfected cells are expanded in number and then reimplanted in the patient. In in vitro gene transfer, the transformed cells are cells growing in culture, such as tissue culture cells, and not particular cells from a particular patient. These "laboratory cells" are transfected, the transfected cells are selected and expanded for either implantation into a patient or for other uses. In vivo gene transfer involves introducing the DNA into the cells of the patient when the cells are within the patient. All three of the broad based categories described above may be used to achieve gene transfer in vivo, ex vivo, and in vitro.

[00038] Mechanical (i.e. physical) methods of DNA delivery can be achieved by direct injection of DNA, such as microinjection of DNA into germ or somatic cells, pneumatically delivered DNA-coated particles, such as the gold particles used in a "gene gun," and inorganic chemical approaches such as calcium phosphate transfection. It has been found that physical injection of plasmid DNA into muscle cells yields a high percentage of cells which are transfected and have a sustained expression of marker genes. The plasmid DNA may or may not integrate into the genome of the cells. Non-integration of the transfected DNA would allow the transfection and expression of gene product proteins in terminally differentiated, non-proliferative tissues for a prolonged period of time without fear of mutational insertions, deletions, or alterations in the cellular or mitochondrial genome. Long-term, but not necessarily

permanent, transfer of therapeutic genes into specific cells may provide treatments for genetic diseases or for prophylactic use. The DNA could be reinjected periodically to maintain the gene product level without mutations occurring in the genomes of the recipient cells. Non-integration of exogenous DNAs may allow for the presence of several different exogenous DNA constructs within one cell with all of the constructs expressing various gene products.

[00039] Particle-mediated gene transfer may also be employed for injecting DNA into cells, tissues and organs. With a particle bombardment device, or "gene gun," a motive force is generated to accelerate DNA-coated high density particles (such as gold or tungsten) to a high velocity that allows penetration of the target organs, tissues or cells. Electroporation for gene transfer uses an electrical current to make cells or tissues susceptible to electroporation-mediated gene transfer. A brief electric impulse with a given field strength is used to increase the permeability of a membrane in such a way that DNA molecules can penetrate into the cells. The techniques of particle-mediated gene transfer and electroporation are well known to those of ordinary skill in the art.

[00040] Chemical methods of gene therapy involve carrier mediated gene transfer through the use of fusogenic lipid vesicles such as liposomes or other vesicles for membrane fusion. A carrier harboring a DNA of interest can be conveniently introduced into body fluids or the bloodstream and then site specifically directed to the target organ or tissue in the body. Liposomes, for example, can be developed which are cell specific or organ specific. The foreign DNA carried by the liposome thus will be taken up by those specific cells. Injection of immunoliposomes that are targeted to a specific receptor on certain cells can be used as a convenient method of inserting the DNA into the cells bearing the receptor. Another carrier system that has been used is the asialoglycoprotein/polylysine conjugate system for carrying DNA to hepatocytes for in vivo gene transfer.

[00041] Transfected DNA may also be complexed with other kinds of carriers so that the DNA is carried to the recipient cell and then resides in the cytoplasm or in the nucleoplasm of the recipient cell. DNA can be coupled to carrier nuclear proteins in specifically engineered vesicle complexes and carried directly into the nucleus.

[00042] Carrier mediated gene transfer may also involve the use of lipid-based proteins which are not liposomes. For example, lipofectins and cytofectins are lipid-based

positive ions that bind to negatively charged DNA, forming a complex that can ferry the DNA across a cell membrane. Fectins may also be used. Another method of carrier mediated gene transfer involves receptor-based endocytosis. In this method, a ligand (specific to a cell surface receptor) is made to form a complex with a gene of interest and then injected into the bloodstream; target cells that have the cell surface receptor will specifically bind the ligand and transport the ligand-DNA complex into the cell.

[00043] Biological gene therapy methodologies usually employ viral vectors to insert genes into cells. The term "vector" as used herein in the context of biological gene therapy means a carrier that can contain or associate with specific polynucleotide sequences and which functions to transport the specific polynucleotide sequences into a cell. The transfected cells may be cells derived from the patient's normal tissue, the patient's diseased tissue, or may be non-patient cells. Examples of vectors include plasmids and infective microorganisms such as viruses, or non-viral vectors such as the ligand-DNA conjugates, liposomes, and lipid-DNA complexes discussed above.

[00044] Viral vector systems which may be utilized in the present invention include, but are not limited to, (a) adenovirus vectors; (b) retrovirus vectors; (c) adeno-associated virus vectors; (d) herpes simplex virus vectors; (e) SV 40 vectors; (f) polyoma virus vectors; (g) papilloma virus vectors; (h) picornavirus vectors; (i) pox virus vectors such as an orthopox, e.g., vaccinia virus vectors or avipox, e.g. canary pox or fowl pox; and (j) a helper-dependent or gutless adenovirus. In the preferred embodiment the vector is an adenovirus.

[00045] Thus, a wide variety of gene transfer/gene therapy vectors and constructs are known in the art. These vectors are readily adapted for use in the methods of the present invention. By the appropriate manipulation using recombinant DNA/molecular biology techniques to insert an operatively linked adiponectin encoding nucleic acid segment into the selected expression/delivery vector, many equivalent vectors for the practice of the present invention can be generated.

[00046] It will be appreciated by those of skill that cloned genes readily can be manipulated to alter the amino acid sequence of a protein. The cloned gene for adiponectin can be manipulated by a variety of well known techniques for in vitro mutagenesis, among others, to

produce variants of the naturally occurring human protein, herein referred to as muteins, that may be used in accordance with the invention.

[00047] The variation in primary structure of muteins of adiponectin useful in the invention, for instance, may include deletions, additions and substitutions. The substitutions may be conservative or non-conservative. The differences between the natural protein and the mutein generally conserve desired properties, mitigate or eliminate undesired properties and add desired or new properties.

[00048] Similarly, techniques for making small oligopeptides and polypeptides that exhibit activity of larger proteins from which they are derived (in primary sequence) are well known and have become routine in the art. Thus, peptide analogs of proteins of the invention, such as peptide analogs of adiponectin that exhibit antagonist activity also are useful in the invention.

[00049] Mimetics also can be used in accordance with the present invention to modulate angiogenesis. The design of mimetics is known to those skilled in the art, and is generally understood to be peptides or other relatively small molecules that have an activity the same or similar to that of a larger molecule, often a protein, on which they are modeled.

[00050] Variations and modifications to the above protein and vectors can be used to increase or decrease adiponectin expression, and to provide means for targeting. For example, adiponectin can be linked with a molecular counterligand for endothelial cell adhesion molecules, such as PECAM-adiponectin, to make these agents tissue specific.

[00051] In one aspect, the present invention provides for a method for the modulation of angiogenesis in a tissue associated with a disease process or condition, and thereby affect events in the tissue which depend upon angiogenesis. Generally, the method comprises administering to the tissue, associated with, or suffering from a disease process or condition, an angiogenesis-modulating amount of a composition comprising adiponectin protein or a nucleic acid vector expressing adiponectin.

[00052] Any of a variety of tissues, or organs comprised of organized tissues, can support angiogenesis in disease conditions including skin, muscle, gut, connective tissue, brain tissue, nerve cells, joints, bones and the like tissue in which blood vessels can invade upon angiogenic stimuli.

[00053] The patient to be treated according to the present invention in its many embodiments is a human patient, although the invention is effective with respect to all mammals. In this context, a "patient" is a human patient as well as a veterinary patient, a mammal of any mammalian species in which treatment of tissue associated with diseases involving angiogenesis is desirable, particularly agricultural and domestic mammalian species.

[00054] Thus, the method embodying the present invention comprises administering to a patient a therapeutically effective amount of a physiologically tolerable composition containing adiponectin protein or nucleic acid vector for expressing adiponectin protein.

[00055] The dosage ranges for the administration of adiponectin protein depend upon the form of the protein, and its potency, as described further herein, and are amounts large enough to produce the desired effect in which angiogenesis is potentiated and the disease symptoms mediated by lack of angiogenesis are ameliorated. The dosage should not be so large as to cause adverse side effects, such as hyperviscosity syndromes, pulmonary edema, congestive heart failure, and the like. Generally, the dosage will vary with the age, condition, sex and extent of the disease in the patient and can be determined by one of skill in the art. The dosage can also be adjusted by the individual physician in the event of any complication.

[00056] A therapeutically effective amount is an amount of adiponectin protein, or nucleic acid encoding for adiponectin, that is sufficient to produce a measurable modulation of angiogenesis in the tissue being treated, i.e., angiogenesis-modulating amount. Modulation of angiogenesis can be measured or monitored by the CAM assay, or by other methods known to one skilled in the art.

[00057] The adiponectin protein or nucleic acid vector expressing such protein can be administered parenterally by injection or by gradual infusion over time. Although the tissue to be treated can typically be accessed in the body by systemic administration and therefore most often treated by intravenous administration of therapeutic compositions, other tissues and delivery means are contemplated where there is a likelihood that the tissue targeted contains the target molecule. Thus, compositions of the invention can be administered intravenously, intraperitoneally, intramuscularly, subcutaneously, intracavity, transdermally, and can be delivered by peristaltic means, if desired.

[00058] The therapeutic compositions containing adiponetic protein or nucleic acid vector expressing the protein can be conventionally administered intravenously, as by injection of a unit dose, for example. The term "unit dose" when used in reference to a therapeutic composition of the present invention refers to physically discrete units suitable as unitary dosage for the subject, each unit containing a predetermined quantity of active material calculated to produce the desired therapeutic effect in association with the required physiologically acceptable diluent, i.e., carrier, or vehicle.

[00059] The compositions are administered in a manner compatible with the dosage formulation, and in a therapeutically effective amount. The quantity to be administered and timing depends on the subject to be treated, capacity of the subject's system to utilize the active ingredient, and degree of therapeutic effect desired.

[00060] Precise amounts of active ingredient required to be administered depend on the judgment of the practitioner and are peculiar to each individual. However, suitable dosage ranges for systemic application are disclosed herein and depend on the route of administration. Suitable regimes for administration are also variable, but are typified by an initial administration followed by repeated doses at one or more hour intervals by a subsequent injection or other administration. Alternatively, continuous intravenous infusion sufficient to maintain concentrations in the blood in the ranges specified for in vivo therapies are contemplated.

[00061] Adiponectin protein and vectors may be adapted for catheter-based delivery systems including coated balloons, slow-release drug-eluting stents, microencapsulated PEG liposomes, or nanobeads for delivery using direct mechanical intervention with or without adjunctive techniques such as ultrasound.

[00062] The adiponectin protein of the invention may be combined with a therapeutically effective amount of another pro-angiogenesis factor and/or vasculogenic agent such as, transforming growth factor alpha (TGF-α), vascular endothelial cell growth factor (VEGF), acidic and basic fibroblast growth factor (FGF), tumor necrosis factor (TNF), and platelet derived growth factor (PDGF).

[00063] Any diseases or condition that would benefit from the potentiation of angiogenesis can be treated by methods of the present invention. For example, stimulation of

angiogenesis can aid in the enhancement of collateral circulation where there has been vascular occlusion or stenosis (e.g. to develop a "biopass" around an obstruction of an artery, vein, or of a capillary system). Specific examples of such conditions or disease include, but are not necessarily limited to, coronary occlusive disease, carotid occlusive disease, arterial occlusive disease, peripheral arterial disease, atherosclerosis, myointimal hyperplasia (e.g., due to vascular surgery or balloon angioplasty or vascular stenting), thromboangiitis obliterans, thrombotic disorders, vasculitis, and the like.

[00064] Other conditions or diseases that can be prevented using the methods of the invention include, but are not necessarily limited to, heart attack (myocardial infarction) or other vascular death, stroke, death or loss of limbs associated with decreased blood flow, and the like. In addition, the methods of the invention can be used to accelerate healing of wounds or ulcers; to improve the vascularization of skin grafts or reattached limbs so as to preserve their function and viability; to improve the healing of surgical anastomoses(e.g., as in re-connecting portions of the bowel after gastrointestinal surgery); and to improve the growth of skin or hair.

[00065] In a preferred embodiment, the methods of the invention are used to treat vascular complications of diabetes.

[00066] The present invention provides therapeutic compositions useful for practicing the therapeutic methods described herein. Therapeutic compositions of the present invention contain a physiologically tolerable carrier together with adiponectin protein or vector capable of expressing adiponectin protein as described herein, dissolved or dispersed therein as an active ingredient. In a preferred embodiment, the therapeutic composition is not immunogenic when administered to a mammal or human patient for therapeutic purposes.

[00067] As used herein, the terms "pharmaceutically acceptable", "physiologically tolerable" and grammatical variations thereof, as they refer to compositions, carriers, diluents and reagents, are used interchangeably and represent that the materials are capable of administration to or upon a mammal without the production of undesirable physiological effects such as nausea, dizziness, gastric upset and the like.

[00068] The preparation of a pharmacological composition that contains active ingredients dissolved or dispersed therein is well understood in the art and need not be limited based on formulation. Typically such compositions are prepared as injectable either as liquid

solutions or suspensions, however, solid forms suitable for solution, or suspensions, in liquid prior to use can also be prepared. The preparation can also be emulsified or presented as a liposome composition. The active ingredient can be mixed with excipients which are pharmaceutically acceptable and compatible with the active ingredient and in amounts suitable for use in the therapeutic methods described herein. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol or the like and combinations thereof. In addition, if desired, the composition can contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents and the like which enhance the effectiveness of the active ingredient.

[00069] The therapeutic composition of the present invention can include pharmaceutically acceptable salts of the components therein. Pharmaceutically acceptable salts include the acid addition salts (formed with the free amino groups of the polypeptide) that are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, tartaric, mandelic and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine and the like.

[00070] Physiologically tolerable carriers are well known in the art. Exemplary of liquid carriers are sterile aqueous solutions that contain no materials in addition to the active ingredients and water, or contain a buffer such as sodium phosphate at physiological pH value, physiological saline or both, such as phosphate-buffered saline. Still further, aqueous carriers can contain more than one buffer salt, as well as salts such as sodium and potassium chlorides, dextrose, polyethylene glycol and other solutes.

[00071] Liquid compositions can also contain liquid phases in addition to and to the exclusion of water. Exemplary of such additional liquid phases are glycerin, vegetable oils such as cottonseed oil, and water-oil emulsions.

[00072] For topical application, the carrier may in the form of, for example, and not by way of limitation, an ointment, cream, gel, paste, foam, aerosol, suppository, pad or gelled stick.

[00073] The amount of the active adiponectin protein (referred to as "agents") used in the invention that will be effective in the treatment of a particular disorder or condition will depend

on the nature of the disorder or condition, and can be determined by standard clinical techniques. In addition, *in vitro* assays such as those discussed herein may optionally be employed to help identify optimal dosage ranges. The precise dose to be employed in the formulation will also depend on the route of administration, and the seriousness of the disease or disorder, and should be decided according to the judgment of the practitioner and each patient's circumstances. However, suitable dosage ranges for administration of agents are generally about 0.01 pg/kg body weight to 1 mg/kg body weight. Effective doses may be extrapolated from dose-response curves derived from *in vitro* or animal model test bioassays or systems.

[00074] Administration of the doses recited above can be repeated. In a preferred embodiment, the doses recited above are administered 2 to 7 times per week. The duration of treatment depends upon the patient's clinical progress and responsiveness to therapy.

[00075] The invention also contemplates an article of manufacture which is a labeled container for providing adiponectin protein of the invention. An article of manufacture comprises packaging material and a pharmaceutical agent contained within the packaging material.

[00076] The pharmaceutical agent in an article of manufacture is any of the compositions of the present invention suitable for providing adiponectin protein and formulated into a pharmaceutically acceptable form as described herein according to the disclosed indications. Thus, the composition can comprise adiponectin protein or a DNA molecule which is capable of expressing the protein.

[00077] The article of manufacture contains an amount of pharmaceutical agent sufficient for use in treating a condition indicated herein, either in unit or multiple dosages.

[00078] The packaging material comprises a label which indicates the use of the pharmaceutical agent contained therein, e.g., for treating conditions assisted by potentiation of angiogenesis, and the like conditions disclosed herein.

[00079] The label can further include instructions for use and related information as may be required for marketing. The packaging material can include container(s) for storage of the pharmaceutical agent.

[00080] As used herein, the term packaging material refers to a material such as glass, plastic, paper, foil, and the like capable of holding within fixed means a pharmaceutical

agent. Thus, for example, the packaging material can be plastic or glass vials, laminated envelopes and the like containers used to contain a pharmaceutical composition including the pharmaceutical agent.

[00081] In preferred embodiments, the packaging material includes a label that is a tangible expression describing the contents of the article of manufacture and the use of the pharmaceutical agent contained therein.

[00082] The references cited throughout this application are herein incorporated by reference.

[00083] It is understood that the foregoing detailed description and the following examples are illustrative only and are not to be taken as limitations upon the scope of the invention. Various changes and modifications to the disclosed embodiments, which will be apparent to those skilled in the art, may be made without departing from the spirit and scope of the present invention. Further, all patents, patent applications and publications cited herein are incorporated herein by reference.

Example 1

Experimental Procedure

Materials

[00084] Phospho-AMPK (Thr172), pan-α-AMPK and phospho-Akt (Ser473), phospho-eNOS (Ser1177) phospho- p42/44 extracellular signal-regulated kinase (ERK) (Thr 202/Tyr 204), ERK, and Akt antibodies were purchased from Cell Signaling Technology (Beverly, Massachusetts). c-Myc tag antibody was purchased from Upstate biotechnology (Lake Placid, New York). eNOS antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, California). Tubulin antibody was purchased from Oncogene (Cambridge, Massachusetts). Recombinant human VEGF was purchased from Sigma (St. Louis, Missouri).

Recombinant proteins

[00085] Mouse adiponectin (amid acids 15-247) was cloned into the bacterial expression vector pTrcHisB (Amersham Pharmacia Biotech, Piscataway, New Jersey). The histidine-tagged proteins were purified using nickel-ion agarose column, monoQ column, and, for removal of lipopolysaccharide, Detoxi-Gel Affinity Pak column (Pierce, Rockford, Illinois).

Cell culture, adenoviral infection and Western blot analysis.

[00086] Human umbilical vein endothelium cells (HUVECs) were cultured in endothelial cell growth medium-2 (EGM-2, San Diego, California). Before each experiment, cells were placed in endothelial cell basal medium-2 (EBM-2, San Diego, California) with 0.5% fetal bovine serum (FBS) for 16 h for serum-starvation. Experiments were performed by the addition of the indicated amount of mouse recombinant adiponectin, VEGF or vehicle for the indicated lengths of time. In some experiments, HUVECs were infected with adenoviral constructs encoding dominant-negative AMPK□2 (28), dominant-negative AKT1 (19) or green fluorescence protein (GFP) at a multiplicity of infection (MOI) of 50 for 24 h. In some experiments, HUVECs were pretreated with LY294002 (10 □M) or vehicle for 1 h before stimulation with adiponectin. Cell lysates were resolved by SDS-PAGE. The membranes were immunoblotted with the indicated antibodies at a 1:1000 dilution followed by the secondary antibody conjugated with horseradish peroxidase (HRP) at a 1:5000 dilution. ECL-PLUS Western Blotting Detection kit (Amersham Pharmacia Biotech, Piscataway, New Jersey) was used for detection.

Migration assay

[00087] Migration activity was measured using a modified Boyden chamber assay. Serum-starved cells were trypsinized and resuspended in EGM-2 with 0.5% FBS. Cell suspension (250 µl, 2.0 x 104 cells/well) were added to the transwell fibronectin-coated insert (6.4 mm diameter, 3.0 µm pore size, Becton Dickinson, Franklin Lakes, New Jersey). Then 750 µl of EGM-2 with 0.5% FBS supplemented with adiponectin (30 µg/ml), VEGF (20 ng/ml) or bovine serum albumin (BSA) (30 µg/ml) were added to lower chamber and incubated for 4 h.

Migrated cells on the lower surface of the membrane were fixed, stained with Giemsa stain solution and eight random microscopic fields per well were quantified. All assays were performed in triplicate.

Tube formation assay

[00088] The formation of vascular-like structures by HUVECs on growth factor-reduced Matrigel (Becton Dickinson) was performed as previously described (28). Twenty-four-well culture plates were coated with Matrigel according to the manufacturer's instructions. Serum-starved HUVECs were seeded on coated plates at 5 x 104 cells/well in EGM-2 with 0.5% FBS containing indicated concentrations of adiponectin, VEGF (20 ng/ml) or BSA (30 μg/ml) and incubated at 37□C for 18 h. Tube formation was observe using an inverted phase contrast microscope (Nikon, Tokyo, Japan). Images were captured with a video graphic system (DEI-750 CE Digital Output Camera, Optronics, Goleta, California). The degree of tube formation was quantified by measuring the length of tubes in 3 randomly chosen fields from each well using the angiogenic activity quantification program (Kurabo, Osaka, Japan). Each experiment was repeated for 3 times.

Mouse angiogenesis assay

[00089] The formation of new vessels in vivo was evaluated by Matrigel plug assay as described previously (28). For these experiments, 400 μl of Matrigel containing adiponectin (100 μg/ml) or vehicle was injected subcutaneously into the abdomen of C57BL mice. Mice were sacrificed 14 days after the injection. The Matrigel plugs with adjacent subcutaneous tissues were carefully recovered by en bloc resection, fixed in 4% paraformaldehyde, dehydrated with 30% sucrose, and embedded in OCT compound (GTI Microsystems, Tempe, Arizona) in liquid nitrogen. Immunohistostaining for CD31 (PECAM-1: Becton Dickinson) were performed on adjacent frozen sections. Primary antibody was used at a 1:50 dilution followed by incubation of secondary antibody (HRP-conjugated anti-rat IgG at a 1:100 dilution). The AEC Substrate Pack (Biogenex, San Ramon, California) was used for

detection. CD31-positive capillaries were counted in 4 randomly chosen low-power (X100) microscopic fields.

Rabbit corneal angiogenesis assay

[00090] Rabbit corneal assay was performed with minor modification as previously described (33). Male New Zealand white rabbits weighing 3.0-3.9 kg were used. Two pockets, about 2x3 mm size and 5 mm apart, were surgically prepared in the cornea extending toward a point 2 mm from the limbus. Hydron pellets, which contain indicated amount of adiponectin, VEGF (100 ng) or PBS and enables the slow release of it (34), were implanted into the pocket. On day 7 after surgery, eyes were photographed and cornea neovascularization was examined in a single blind manner. The angiogenic activity was evaluated on the basis of the number and growth rate of newly formed capillaries. An angiogenic score was calculated (vessel density x distance from limbus) (32). A density value of 1 corresponded to 0-25 vessels per cornea, 2 from 25-30, 3 from 50-75, 4 from 75-100 and 5 for >100 vessels.

Statistic Analysis

[00091] Data are presented as mean ± SE. Differences were analyzed by Student's unpaired t test. A level of $P<0.05$ was accepted as statistically significant.

Results

Adiponectin accelerates vascular structure formation in vitro

[00092] We first examined whether adiponectin affected endothelial cell differentiation into capillary-like structure when HUVECs were plated on a Matrigel matrix. Treatment with a physiological concentration of adiponectin promoted the formation of capillary-like tubes in a manner similar to VEGF (Fig. 1A). Quantitative analyses of tube structure length revealed a trend toward increased tube length in the VEGF-treated cultures

relative to adiponectin, but this was not statistically significant (Fig. 1B). To test whether adiponectin modulated the endothelial migration, a modified Boyden chamber assay was performed. Adiponectin significantly stimulated HUVEC migration, as did VEGF (Fig. 1C). Quantitative analyses revealed a trend toward greater migration with VEGF compared to adiponectin, but this was not statistically significant. Adiponectin also induced the endothelial migration in a cell-wounding assay (N. Ouchi et al., unpublished data). These result suggest that adiponectin promotes pro-angiogenic cellular responses in endothelial cells.

[00093] Adiponectin induces the phosphorylation of AMPK, Akt and eNOS Endothelial AMPK signaling is associated with the regulation of angiogenesis under certain conditions (28). Therefore, to test whether adiponectin induces AMPK signaling in endothelial cells, cultured HUVECs were incubated with adiponectin, and AMPK phosphorylation at Thr 172 of α subunit was assessed by Western blot analyses. Treatment of HUVECs with adiponectin enhanced the phosphorylation of AMPK in a time-dependent manner with maximal AMPK phosphorylation occurring at 15 minutes (Fig. 2A). Akt plays important roles in the angiogenic response to several growth factors and cytokines(18). Therefore, the effect of adiponectin on the activating phosphorylation of Akt at Ser 473 was investigated. Adiponectin treatment led to a time-dependent increase in Akt phosphorylation (Fig. 2A). In contrast to these signaling protein kinases, adiponectin treatment had no effect on the phosphorylation of ERK at Thr 202/Tyr 204 (Fig. 2A). Both AMPK and Akt can phosphorylate eNOS at Ser 1179 (22,23,35,36). Therefore, eNOS phosphorylation was examined in these cultures. Adiponectin stimulation promoted a time-dependent increase in eNOS phosphorylation at Ser 1179, but had no effect on eNOS protein levels (Fig. 2A).

[00094] The regulation of eNOS by mitogen-stimulated phosphorylation is complicated by the possibility of AMPK-Akt cross-talk (28,37). To examine the relative contribution of AMPK and Akt to the regulation of adiponectin-induced phosphorylation of eNOS, HUVECs were transduced either with an adenoviral vector expressing a c-Myc-tagged dominant-negative mutant of AMPK (ad-dnAMPK) or dominant-negative Akt (ad-dnAkt). Transduction with ad-dnAMPK suppressed adiponectin-induced AMPK and eNOS

phosphorylation (Fig. 2B). Transduction with ad-dnAMPK also blocked adiponectin-induced phosphorylation of Akt suggesting signaling cross-talk between these two protein kinases (Fig. 2B). Of note, transduction with ad-dnAkt suppressed the adiponectin-induced phosphorylation of eNOS without altering that of AMPK (Fig. 2C). These data indicated that Akt is a downstream kinase of AMPK and that Akt mediates eNOS phosphorylation downstream from adiponectin/AMPK.

AMPK and Akt signaling are required for adiponectin-stimulated migration and differentiation

[00095] To test whether AMPK and Akt signaling participate in adiponectin-stimulated endothelial differentiation and migration, HUVECs were infected with ad-dnAMPK or ad-dnAkt and evaluated in tube formation and Boyden chamber assays, respectively. Transduction with either ad-dnAMPK or ad-dnAkt suppressed adiponectin-induced endothelial tube structure formation to basal levels (Fig. 3, A and B). In contrast, VEGF-stimulated differentiation was blocked by transduction with ad-dnAkt, but not by transduction with ad-dnAMPK (Fig. 3B). Transduction with ad-dnAMPK and ad-dnAkt had no effect on non-stimulated, basal tube formation (Fig. 3B). Adiponectin-stimulated endothelial migration was also significantly suppressed by transduction with either ad-dnAMPK or ad-dnAkt (Fig. 3C). In contrast, transduction with ad-dnAkt blocked VEGF-stimulated migration, while transduction with ad-dnAMPK had no effect (Fig. 3C). Transduction with ad-dnAMPK and ad-dnAkt had no effect on the basal migration rate (Fig. 3C). These results indicated that both AMPK and Akt signals are required for adiponectin-induced endothelial migration and differentiation, whereas only Akt signaling participates in these endothelial cell responses to VEGF.

Role of PI3-kinase signaling in adiponectin-induced angiogenic response

[00096] Akt is activated by many growth factors and cytokines in a PI3-kinase-dependent manner (18). To investigate whether PI3-kinase signal is involved in adiponectin-induced angiogenic signaling pathway, HUVECs were incubated with PI3-kinase inhibitor, LY294002 in the absence or presence of adiponectin. Brief treatment with LY294002 abolished

adiponectin-stimulated tube formation and migration (Fig. 4, A and B). Adiponectin-stimulated the phosphorylation of Akt and eNOS was blocked by treatment with LY294002, while LY294002 treatment had no effect on AMPK phosphorylation (Fig. 4C). These data indicate that PI3-kinase is a critical for adiponectin-induced angiogenic cell responses and that PI3-kinase functions upstream from the Akt-eNOS regulatory axis in adiponectin-stimulated endothelial cells.

Adiponectin promotes vessel growth in vivo

[00097] To examine the in vivo effect of adiponectin on angiogenesis, mouse Matrigel plugs and rabbit corneal assays were performed. In the Matrigel plugs assay, endothelial cell infiltration of the plugs was assessed by immunohistochemical analysis of CD31-positive cells (Fig. 5A). Quantitative analyses of histological sections revealed that plugs containing adiponectin displayed a significantly higher density of CD31-positive cells compared with controls (Fig. 5B). In addition, the angiogenic activity of adiponectin was essential in a rabbit corneal assay. Neovascularization in corneal implants containing adiponectin was markedly accelerated compared with controls (Fig. 5, C and D). The stimulatory effect of adiponectin was comparable with that of VEGF in this model (Fig. 5, C and D). These data show that adiponectin can promote neovascularization in vivo.

Discussion

[00098] This study identifies the promotion of blood vessel growth as a new role for the adipocytokine adiponectin. Proangiogenic activity was demonstrated in two well-established models of angiogenesis, the mouse Matrigel plug and rabbit corneal assays. The ability of adiponectin to stimulate angiogenesis is likely due, at least in part, to its ability to promote endothelial cell migration and stimulate the differentiation of these cells into capillary-like structures.

[00099] Adiponectin functions as an AMPK activator in multiple cell types (29-32,38). Recently, we reported that endothelial AMPK signaling is essential for angiogenesis under conditions of hypoxia, but dispensable in normoxic cells. Here it is shown that AMPK activation by adiponectin can activate angiogenic cellular responses in normoxic endothelial cells. Furthermore, it is shown that cross-talk between AMPK and Akt protein kinases is required several cellular responses downstream of adiponectin including the activating phosphorylation of eNOS at Ser 1179. Several recent reports have demonstrated the importance of AMPK-Akt cross-talk (28,37). While both Akt and AMPK are reported to directly phosphorylate eNOS (22,23,35,36), our study found that transduction with either ad-dnAMPK or ad-dnAkt effectively blocked adiponectin-induced eNOS phosphorylation. Both of these reagents also suppressed adiponectin-stimulated endothelial cell migration and differentiation. Furthermore, inhibition of AMPK signaling suppressed adiponectin-induced Akt phosphorylation, suggesting that Akt functions downstream of AMPK in adiponectin-stimulated endothelial cells (Fig. 6). Importantly, the PI3-kinase inhibitor LY294002 blocked adiponectin-stimulated cell migration, differentiation and Akt and eNOS phosphorylation, without altering the phosphorylation status of AMPK. These data suggest that the pro-angiogenic effects of adiponectin-stimulated AMPK activity are due, in large part, to an activation of Akt signaling under these conditions. Although we cannot exclude the possibility that AMPK directly phosphorylates eNOS, the data is most consistent with a model that comprises an adiponectin-AMPK-PI3-kinase-Akt-eNOS signaling axis under the conditions of our assays (Fig. 6).

[000100] The hypothesis that AMPK functions upstream of Akt signaling is consistent with data obtained from studies in other systems. For example, it has been shown that the AMPK stimulator 5-aminoimidazole-4-carboxamide riboside enhances insulin-stimulated activation of IRS-1-associated PI3-kinase in C2C12 myocytes (39). Furthermore, adiponectin-deficient mice exhibit severe diet-induced insulin resistance that coincides with a reduction of muscle IRS-1-associated PI3-kinase activity (14). Conversely, adiponectin stimulates IRS-1-associated PI3-kinase activity in C2C12 myocytes (14), and adiponectin treatment increases insulin-stimulated Akt phosphorylation in the skeletal muscle of adiponectin-treated lipoatrophic mice (40).

[000101] Plasma adiponectin levels are low in patients with type 2 diabetes (9). Low levels of adiponectin expression have also been observed in the visceral fat of diabetic fa/fa Zucker rats in comparison with lean rats (41). Clinically, collateral vessel development is impaired in diabetic patients including those with myocardial and limb ischemia (42,43) and, in animal models, there is an impaired angiogenic response following ischemic injury in nonobese diabetic mice and obese diabetic fa/fa Zucker rats (44,45). Therefore, low adiponectin levels may contribute the impaired collateral growth in diabetic states. Taken together, these data suggest that the exogenous supplementation of adiponectin is useful treatment for vascular complications of diabetes and other ischemic diseases.

[000102] It will be apparent to those skilled in the art that various modifications and variations can be made to the present invention without departing from the spirit and scope of the invention. Thus, it is intended that the present invention cover the modifications and variations of this invention provided they come within the scope of the appended claims and their equivalents.

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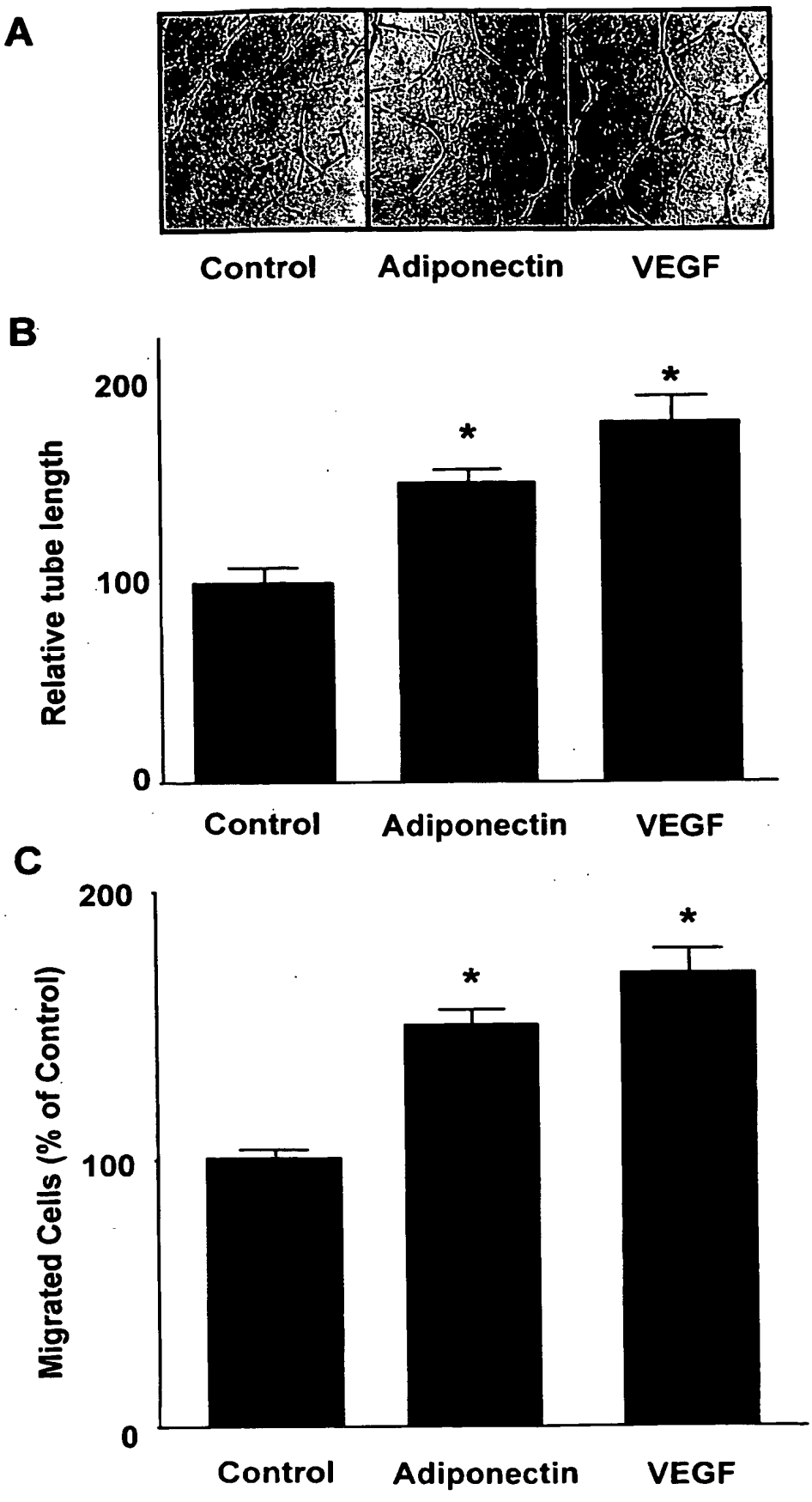
CLAIMS

WHAT IS CLAIMED IS:

1. A method for stimulating angiogenesis in a tissue associated with a disease condition comprising administering to said tissue an angiogenesis stimulating amount of a pharmaceutical composition comprising adiponectin protein or a nucleotide sequence encoding for said protein.
2. The method of claim 1, wherein said tissue has abnormal circulation.
3. The method of claim 1, said disease condition is diabetes.
4. The method of claim 1, wherein said administering comprises intravenous, transdermal, intrasynovial, intramuscular, or oral administration.
5. A pharmaceutical composition for stimulating angiogenesis in a target mammalian tissue comprising a gene transfer vector containing a nucleic acid, said nucleic acid having a nucleic acid segment encoding for adiponectin protein and a pharmaceutically acceptable carrier or excipient.
6. A pharmaceutical composition for stimulating angiogenesis in a target mammalian tissue comprising a therapeutic amount of adiponectin protein, and a pharmaceutically acceptable carrier or excipient.
7. An article of manufacture comprising packaging material and a pharmaceutical composition contained within said packaging material, wherein said pharmaceutical composition is capable of stimulating angiogenesis in a tissue associated with a disease condition, wherein said packaging material comprises a label which indicates that said pharmaceutical composition can be used for treating disease conditions by stimulating angiogenesis, and wherein said pharmaceutical composition comprises adiponectin protein or a vector containing a DNA segment encoding said protein.

ABSTRACT

We have surprisingly discovered that adiponectin regulates angiogenesis. As a result of this discovery, the present invention provides methods for stimulation of angiogenesis in tissues using adiponectin. In a preferred embodiment, the methods of the invention are used to treat vascular complications of diabetes.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Control
Adiponectin
VEGF
B
Relative tube length
200
100
0
*
*
Control
Adiponectin
VEGF
C
Migrated Cells (% of Control)
200
100
0
*
*
Control
Adiponectin
VEGF

Fig. 1

A

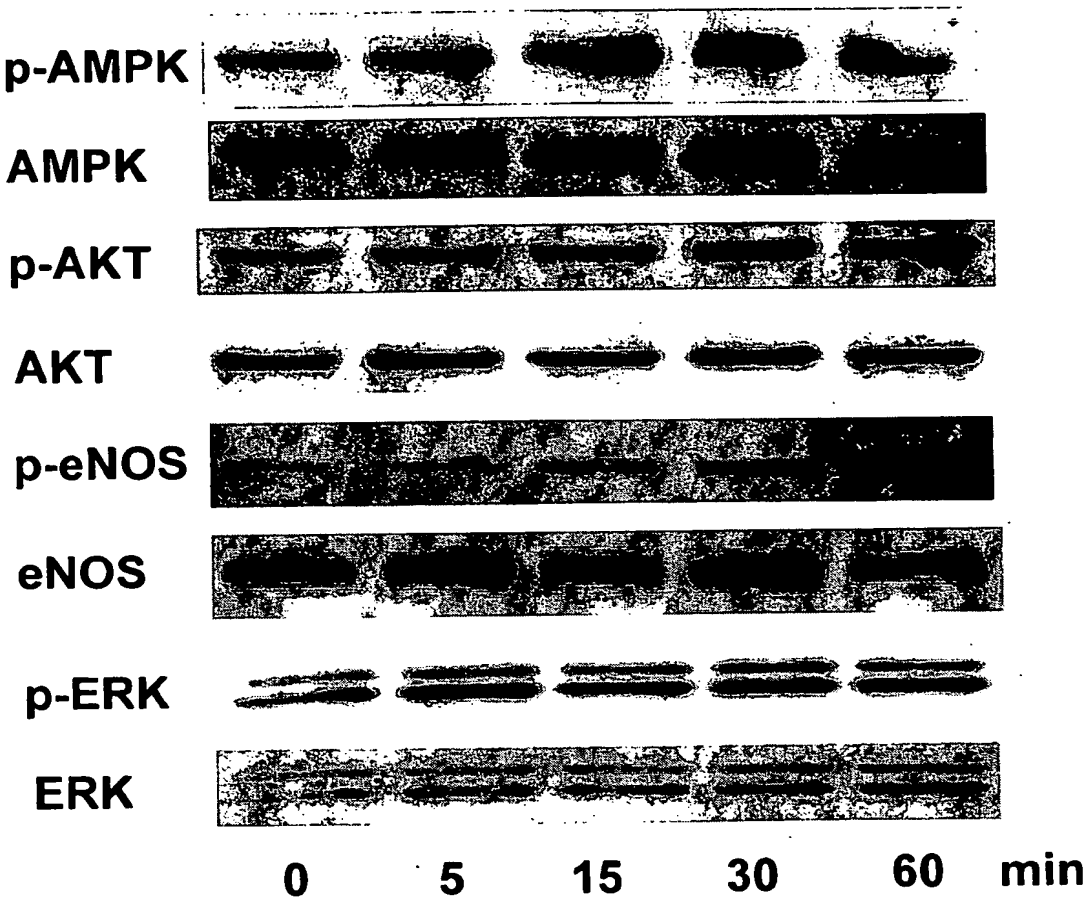
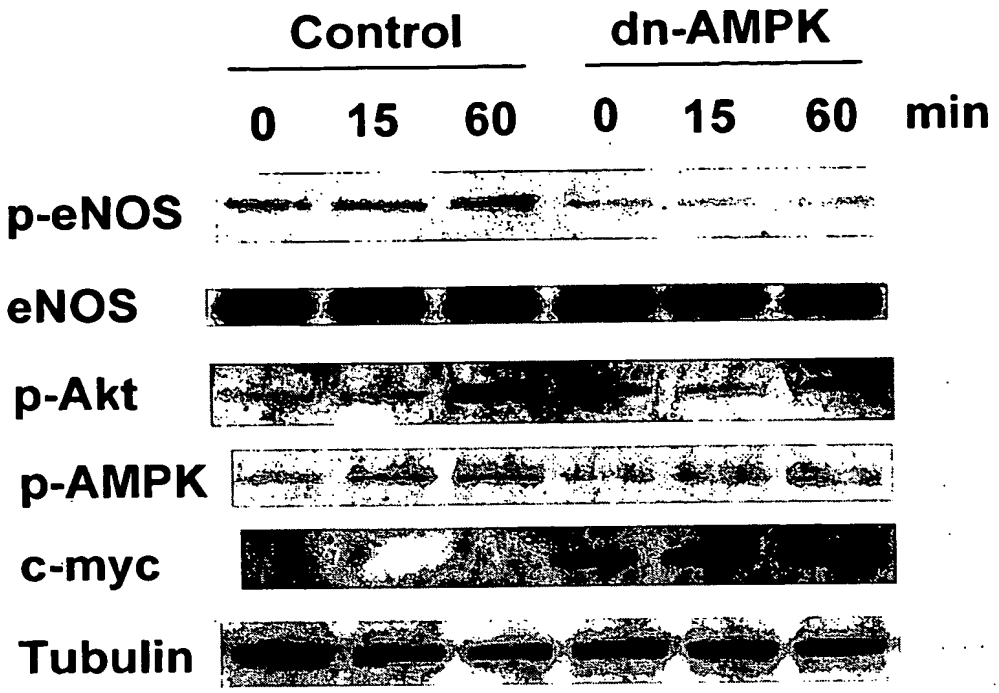


Fig. 2

B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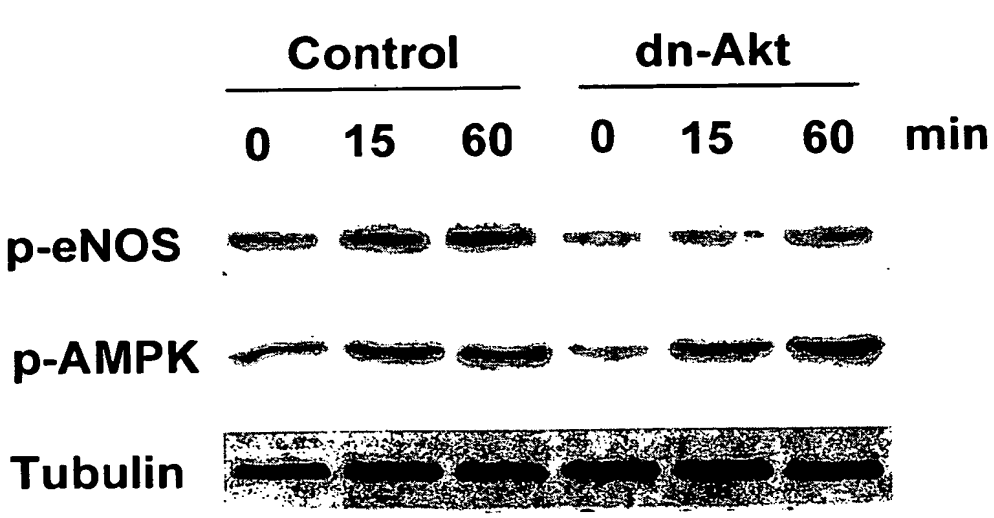
eNOS
p-Akt
p-AMPK
c-myc
Tubulin

C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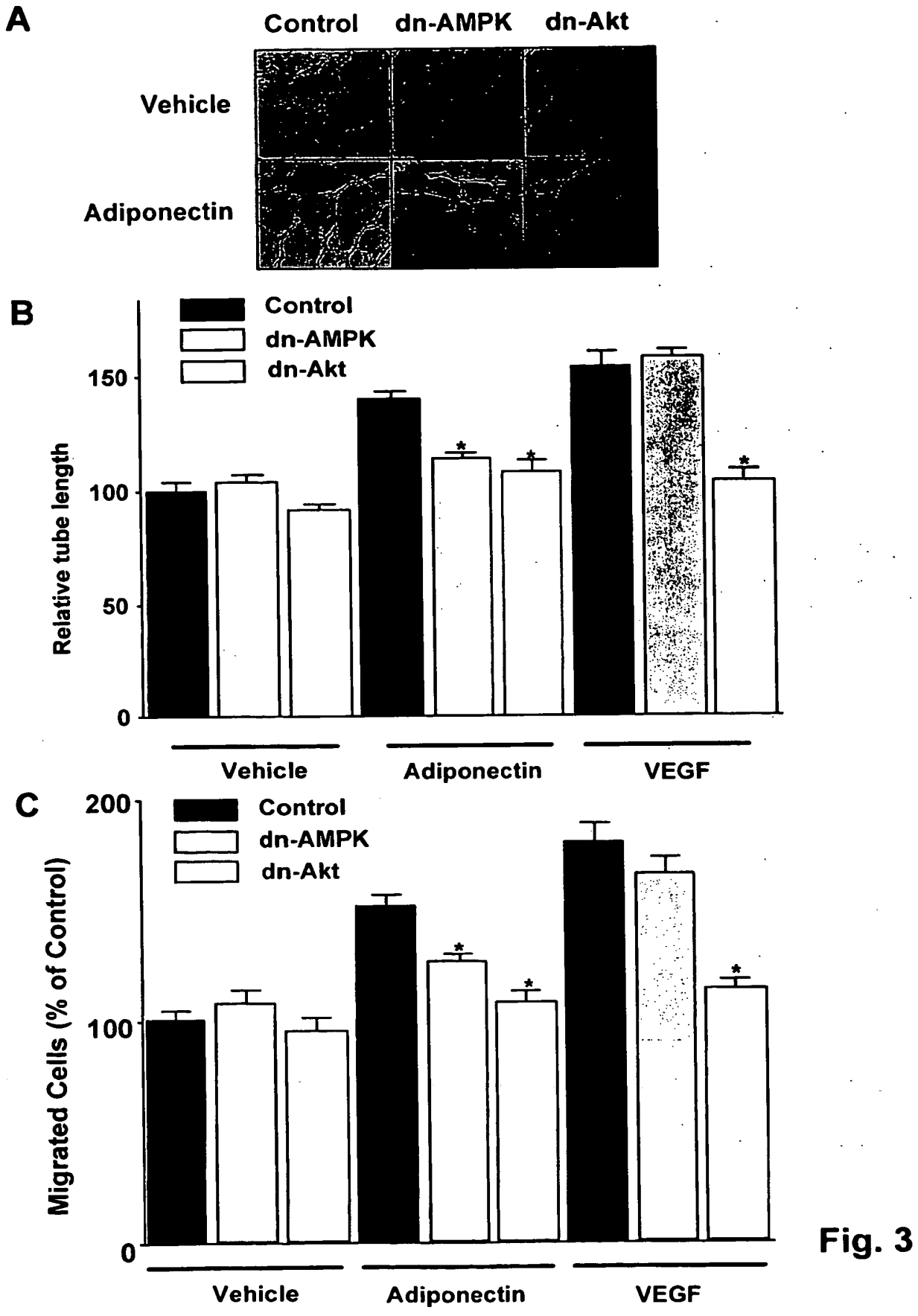
dn-Akt
0 15 60 0 15 60 min
p-eNOS
p-AMPK
Tubulin

Fig. 2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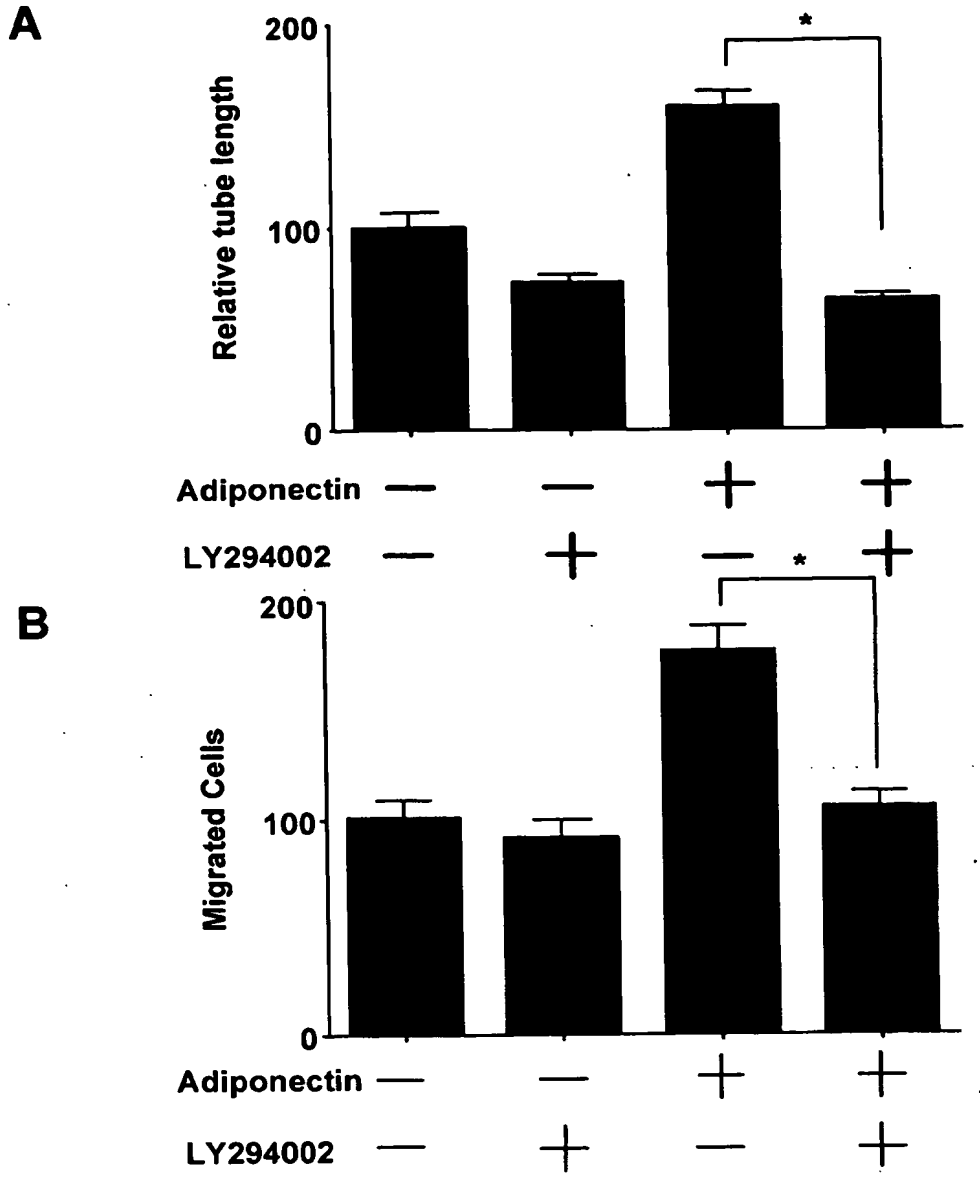
Control
dn-AMPK
dn-Akt
Vehicle
Adiponectin
B
Control
dn-AMPK
dn-Akt
Relative tube length
150
100
50
0
*
Vehicle
Adiponectin
VEGF
C
Control
dn-AMPK
dn-Akt
Migrated Cells (% of Control)
200
100
0
*
Vehicle
Adiponectin
VEGF

Fig. 3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100
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Relative tube length
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LY294002 — + — +
B
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100
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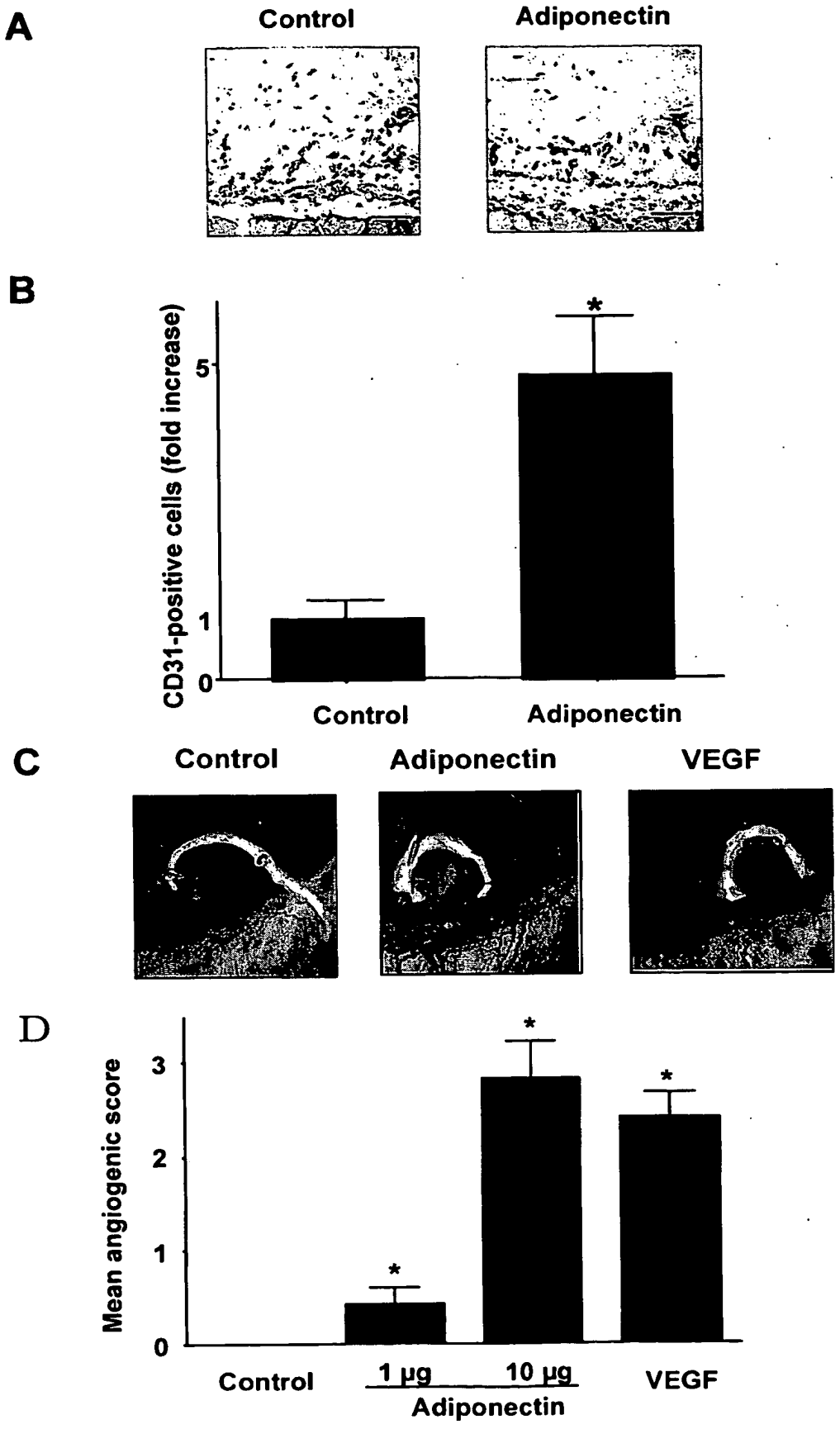
*
Adiponectin — — + +
LY294002 — + — +
C
Control
LY294002
0 15 60 0 15 60 min
p-eNOS
p-Atk
p-AMPK
Tubulin

Fig. 4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Control
Adiponectin
B
CD31-positive cells (fold increase)
5
1
0
*
Control
Adiponectin
C
Control
Adiponectin
VEGF
D
Mean angiogenic score
3
2
1
0
*
*
*
Control
1 µg
10 µg
Adiponectin
VEGF

Fig. 5

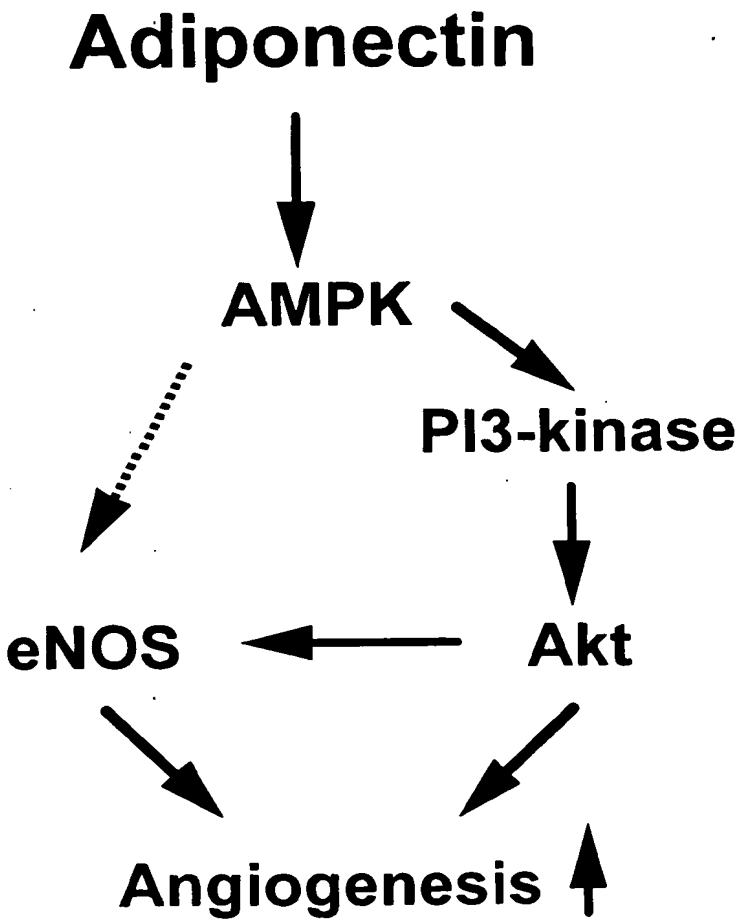

Fig. 6

Docket No. 701586-054550-P *PATENT*

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: Kenneth Walsh and Noriyuki Ouchi
Application No.: To be assigned
Filed: To be assigned
For: METHOD AND COMPOSITION FOR STIMULATION OF ANGIOGENESIS

Mail Stop Provisional Application
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

APPLICATION DATA SHEET
37 C.F.R. § 1.76

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3. Application information

Title of Invention: METHOD AND COMPOSITION FOR STIMULATION OF ANGIOGENESIS

Docket number assigned to this application: 701586-054550-P

Suggested Classification: Class:
Subclass:
Technology Center to which subject matter is assigned:

In re application of:	Kenneth Walsh and Noriyuki Ouchi
Application No.:	To be assigned
Filed:	To be assigned
For:	METHOD AND COMPOSITION FOR STIMULATION OF ANGIOGENESIS

Total number of drawing sheets: 7

Type of application: Provisional

Secrecy order under § 5.2: N/A

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Extent of interest of assignee in application: entire

Date: 10/9/03

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Document made available under the Patent Cooperation Treaty (PCT)

International application number: PCT/US04/033178

International filing date: 08 October 2004 (08.10.2004)

Document type: Certified copy of priority document

Document details: Country/Office: US
Number: 60/510,057
Filing date: 09 October 2003 (09.10.2003)

Date of receipt at the International Bureau: 17 March 2005 (17.03.2005)

Remark: Priority document submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b)

World Intellectual Property Organization (WIPO) - Geneva, Switzerland
Organisation Mondiale de la Propriété Intellectuelle (OMPI) - Genève, Suisse